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Extracellular enzyme activity of model cold-adapted bacteria and Arctic sea-ice microbial communities under subzero hypersaline conditions

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ABSTRACT: Bacterially produced extracellular enzymes (EEs) play an important role in the cycling of organic matter in the marine environment, breaking down large compounds to those small enough to be transported across the cell membrane. EEs may play an especially important role within the brines of sea ice, as freezing concentrates both bacteria and organic materials into brine pockets, leading to higher encounter rates between EEs and their substrates. However, whether EEs are able to perform under the extreme conditions of sea-ice brines, particularly during winter, is unknown. Here, we characterized EE activity (EEA) of leucine aminopeptidase produced by the psychrophilic bacterium *Colwellia psychrerythraea* strain 34H and the cold-tolerant *Psychrobacter* strain 7E, under analogue sea-ice conditions using a standard fluorescence-based activity assay. EEs produced by the psychrophile were active at the most extreme conditions tested, i.e. temperature of −8°C and salt concentration of 120 ppt, with activity enhanced if the EEs concerned were produced under subzero hypersaline conditions. EEs produced by the *Psychrobacter* strain were less cold- and salt-active. When high-latitude Arctic samples of sea-ice brine, under-ice water, and the sea-surface microlayer were analyzed using the same assay after a freeze–thaw cycle, EEA was highest in the sea-ice samples, with activity at −10°C and salinity of 142 ppt. Overall, these results indicate that EEA can contribute to the degradation of organic material in sea ice through winter, likely sustaining microbial communities in brine pores in the process and altering the nature of organic material released at spring melt.

KEY WORDS: Arctic marine environment · Carbon cycle · *Colwellia psychrerythraea* strain 34H · Enzymatic activity · Psychrophiles · Sea ice · *Psychrobacter* strain 7E

1. INTRODUCTION

As a result of active microbial communities and freeze-concentration effects, the interstitial network of brine channels and pores within sea ice contains concentrations of dissolved organic matter (DOM) that can greatly exceed those of underlying seawater (Thomas et al. 2001, Underwood et al. 2013). Heterotrophic bacteria within sea ice rely on this organic material for activity and growth, yet the majority of marine organic material is considered too large for cellular uptake (>600 kDa; Chróst 1991). To facilitate degradation of DOM to a utilizable size, bacteria commonly produce extracellular enzymes (EEs), hydrolytic proteins within the periplasmic space of the cell, at the cell surface ('cell-attached EEs') or released into the environment ('free EEs') (Chróst 1991, Arnosti 2011).

EE activity (EEA) has long been recognized as the rate-limiting step in the bacterial cycling of high molecular weight organic material (Chróst 1991); understanding EEA is thus key to understanding broader organic cycling within the environment. While many studies have characterized bacterial EEA within Arctic marine environments, with high rates of organic hydrolysis demonstrated throughout

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the water column (Vetter & Deming 1994, Steen & Arnosti 2013), especially on sinking aggregates (Huston & Deming 2002, Kellogg & Deming 2014), fewer measurements have been made on sea ice (Helmke & Weyland 1995, Huston et al. 2000, Deming 2007, Yu et al. 2009) or on sea-ice isolates at or near *in situ* conditions of cold temperature and hypersalinity (Groudieva et al. 2004, Huston et al. 2004, Q. Wang et al. 2005, X. Wang et al. 2018).

Sea ice experiences low temperatures, from 0 to below −30°C, which slow chemical reactions and impart structural changes to proteins, limiting activity. At the same time, organisms in sea ice can experience wide salinity ranges, with salt concentrations of 0 to >230 ppt, altering protein interactions with surrounding water molecules due to charge differences (Ortega et al. 2011). In combination, low temperature and high salt not only increase the viscosity of sea-ice brine, limiting diffusional processes (Cox & Weeks 1975), but also dramatically reduce water activity within the brines, a known challenge for enzymes. Enzymes in sea ice must also contend with small-scale salinity changes as atmospheric temperatures drive fluctuations in brine volume and salinity on hourly to seasonal cycles (Ewert & Deming 2013). Extremophilic bacteria, such as psychrophiles (those with maximum growth rates below 15°C; Morita 1975) and halophiles (those growing optimally at salinities matching or above seawater; Kushner & Kamekura 1988), have commonly been studied for their potential to produce enzymes that operate under an extreme condition relevant to an environmental or industrial process. Such investigations have produced an understanding of enzymes that operate at freezing temperatures (Huston et al. 2004) or up to saturating salt conditions (Ortega et al. 2011), but rarely have enzymes been investigated under concurrent temperature and salinity extremes (Karan et al. 2020) that characterize sea-ice brines.

How EEs function under such concurrent extremes and in response to freeze–thaw cycles can inform community ecology within sea-ice brines. Although sea-ice brines may have higher concentrations of DOM than underlying seawater due to freeze-concentration effects alone, high primary productivity and exopolysaccharide production by algal assemblages within the bottom centimeters of the ice (Krembs et al. 2002, Meiners et al. 2003) provide new input of DOM. Previous studies have indicated that DOM within sea-ice brines contains a higher fraction of bio-available material compared to underlying seawater (Kähler et al. 1997, Amon et al. 2001), implying that enzymatic degradation of DOM may contribute to increased bioavailability for heterotrophic organisms inhabiting the ice. EEA in sea ice also has implications for DOM cycling in the waters of seasonally ice-covered seas. At the air–sea interface, where sea ice will form and melt, a thin (<1 mm) gelatinous film called the sea-surface microlayer (SML) serves both as a source of material that freezes into new ice and as the collection reservoir for biopolymers released from melting ice (Galgani et al. 2016, Wurl et al. 2017, Engel et al. 2018). EEA within sea-ice brines may thus influence the nature of organic material within the SML during ice melt by releasing more bioavailable material into it, while EEs that retain activity through the temperature and salinity shifts of melting ice and are released from the ice may continue to degrade organic material within the SML.

To understand how concurrent temperature and salinity extremes and rapid changes of conditions due to freezing or thawing impact enzyme activity, we measured EEA of 2 psychrophilic bacteria, *Colwellia psychrerythraea* strain 34H (*Cp*34H) and *Psychrobacter* strain 7E (*P*7E), in laboratory experiments. These measurements were complemented by EEA experiments using natural communities from samples of Arctic seawater, SML, and sea ice across co-varying temperature and salinity conditions. Taken together, these measurements survey the extremes of EEA within cold marine environments and depict how EEA may function at the freeze–thaw boundary of a polar ocean to influence organic matter composition.

2. MATERIALS AND METHODS

2.1. Cultured bacterial growth and harvest

Glycerol-protected stocks of *Cp*34H and *P*7E were reconstituted from −70°C freezer storage. Strain *Cp*34H was originally isolated from Arctic marine sediments, but as strains of *Colwellia psychrerythraea* can be found in sea ice (Boetius et al. 2015), *Cp*34H is often used as a model marine psychrophile, including in the context of cold-active enzymes (Huston et al. 2000, 2004, Methé et al. 2005, Bauvois et al. 2008). *Cp*34H has a growth range from −12 to +18°C (at 35 ppt) and from 17.5 to 70 ppt (at −1°C) (Huston et al. 2000, Wells & Deming 2006), while *P*7E, isolated from upper sea-ice brine, has a similar growth range in temperature (−8 to +25°C) but a more extreme range in salinity (17.5 to 125 ppt) (Ewert & Deming 2014).

Cells were grown in half-strength 2216 Difco Marine Broth, adjusted to full-strength seawater salinity

(35 ppt) using artificial seawater (ASW: 0.4 M NaCl, 9 mM KCl, 26 mM $MgCl_2$, 28 mM $MgSO_4$, and 5 mM TAPSO buffer), at the targeted growth temperature (−8, −4, −1, or +8°C). For experiments manipulating growth salinity, full-strength culture broth was amended with an NaCl solution in distilled (DI) water to achieve the desired salt concentration (35, 55, or 70 ppt) while maintaining half-strength organics. All cells for growth-condition experiments were subcultured twice under the desired conditions, before use in the experiments, by transferring 100 µl of turbid culture into fresh media. This approach provided for at least 8 generations under the desired conditions and was taken to ensure that the cells used reflected those conditions.

When a culture reached mid-log stage as determined by optical density (OD), verified earlier based on cell counts using epifluorescent microscopy (roughly 10^6 cells ml^{-1}), an aliquot was removed and fixed with 2% final concentration formaldehyde for counting by epifluorescence microscopy (see Section 2.5). Counts were scaled to the experimental volume. The remaining sample was centrifuged at slow speed (40 min at 400 × *g*, maintaining temperature between 2 and 6°C), and the supernatant was filtered using a 0.2 µm syringe filter to remove cells. Cell-free supernatant was confirmed periodically by epifluorescence microscopy.

2.2. Laboratory enzyme activity assays

Rates of EEA were determined using a standard fluorogenic substrate assay as originally described by Hoppe (1983). Briefly, an artificial substrate with a fluorescent tag was added to samples and allowed to incubate under experimental conditions with periodic measurements taken for fluorescence. As EEs cleave the artificial substrate, the fluorescent tag is released and the resulting change in fluorescence is measured to indicate rate of enzyme activity. We chose to measure leucine aminopeptidase activity using the fluorogenic substrate L-leucine-7-amido-4-methylcoumarin hydrochloride (MCA-L, Sigma). MCA-L is a common artificial substrate for EEA assays in marine environments, with aminopeptidases often performing the most rapid, observable hydrolysis among field experiments. Additionally, the leucine aminopeptidase of *Cp*34H is well characterized (Huston et al. 2004, Bauvois et al. 2008), and an NCBI BLAST survey using the sequence of leucine aminopeptidase of *Cp*34H suggests that a similar enzyme is present in the genome of *P*7E. MCA-L was dissolved in methyl cellosolve (monomethyl ether, Sigma) to make a 10 mM stock solution from which further dilutions were made in DI water according to experimental plans.

Harvested cell-free supernatant was added to 96-well plates set on ice to prevent warming. Wells were pre-loaded with either DI water or a NaCl solution in DI water and with MCA-L in methyl cellosolve diluted in ASW such that these solutions together achieved desired experimental salinities (17.5, 35, 55, 70, 90, or 120 ppt salinity) and a saturating MCA-L concentration (250 µM, resulting in 2.5% v/v final concentration of methyl cellosolve in samples). The latter was determined by a saturation curve performed at each experimental temperature and for both *Cp*34H and *P*7E. Each treatment was performed in triplicate.

After the 96-well plate was loaded with 3 samples for each treatment, fluorescence was measured in triplicate using an OD spectrophotometer (Spectramax M2, Molecular Devices) set at optimal wavelength for the MCA tag (excitation 380 nm, emission 440 nm) and zeroed against a blank (cell supernatant without MCA-L). Free MCA compound (7-amido-4-methylcoumarin hydrochloride) was measured in cell-free solution at all salinities and across endpoint temperatures to confirm that there was no confounding drift in fluorescence. Time points were selected based on estimated rate of activity and confined to a total period of 24–48 h. This sampling frame is commonly used among EEA assays that include the cell-attached fraction in order to ensure that no other metabolic response to artificial substrate obscures measurements; it was chosen to allow comparison of rates between these experiments, later field experiments, and literature. Plates were kept at their experimental temperatures until measured, then immediately returned to that temperature. Measurement time was short (<3 min), but to verify that no significant warming had occurred, duplicates were made of selected treatments and left at their experimental temperature for end-point comparison; no warming effects were observed.

2.3. Field experiments

Sea-ice cores were collected in the central Arctic Ocean at latitudes of 88–90° N during the Microbiology-Ocean-Cloud-Coupling in the High Arctic (MOCCHA) expedition aboard the icebreaker 'Oden' in August and September of 2018, as described by Torstensson et al. (2021). After collection,

the bottom 10 cm sections of the cores were returned to the ship and thawed directly at temperatures of 4 to 10°C in a shipping container modified to serve as an on-board cold lab. The melted sections from one field site were then pooled (n = 2–3) and the meltwater was subsampled for EEA measurement within 24 h. Sea-ice properties were characterized (see Torstensson et al. 2021), but core temperatures fell within the range of 0 to −4.8°C, with a narrower range of −0.85 to −2.67°C for the bottom 10 cm. Bulk salinity was measured using a handheld refractometer and ranged between 2 and 4 ppt.

For comparison to the sea-ice cores, surrounding ice-related environments were also sampled: seawater was collected from approximately 1 m below the ice using a hand pump, and the SML was sampled from an open lead near the ship using a glass plate technique, either by hand or using an automated catamaran (Ribas-Ribas et al. 2017). Both seawater and SML water were returned to the ship in dark insulated containers and stored in a refrigerator at 4°C before being sub-sampled for EEA within 12 h. Sample salinity was again measured using a handheld refractometer, with SML salinity at 8 ppt and seawater salinity between 31 and 33 ppt.

EEA was measured in the melted sea-ice, SML, and seawater samples at temperatures near *in situ*, on warmed seawater samples, and on frozen SML and re-frozen sea-ice samples to investigate EEA after a freeze–thaw cycle. Sample aliquots were distributed into clean glass tubes and mixed with a saturating concentration of MCA-L (250 µM), which was determined from a saturation curve generated from sea-ice samples. Samples were incubated at near *in situ* temperatures (−1°C for melted sea ice and SML, using an upright freezer; −1.8°C for under-ice water, using a temperature-controlled shipping container), then refrozen (−1.8°C for SML in the temperature-controlled shipping container; −10°C for sea ice and SML in an upright freezer) or warmed (6°C for seawater, in a temperature-controlled shipping container). Fluorescence was measured using a Trilogy Laboratory Fluorometer (Turner) at 4–6 time points over a 24–48 h period. For frozen samples, EEA was measured either as endpoints or over time points after freezing and then re-thawing. Endpoint samples were first spiked with MCA-L and measured for fluorescence, then thawed after a 10 d incubation period and measured to observe change in fluorescence. Freeze-thaw samples were first frozen for 10 d, then thawed, distributed in aliquots, spiked with MCA-L, and measured for EEA over 24 h. Prior to distributing aliquots for enzyme assay, subsamples from all samples were fixed with formaldehyde for subsequent bacterial counts to enable calculation of cell-specific EEA rates. For re-freezing experiments, the expected brine salinity was calculated based on Cox & Weeks (1983).

2.4. Rate calculation

Technical replicate fluorescence measurements were fit with a linear approximation using least squares regression to find average change in fluorescence. Using these slopes, enzyme activity rates were calculated in units of nanomoles of MCA liberated per hour per cell by applying conversion values determined from a free-substrate concentration curve. These curves were measured at endpoint salinities (17.5 and 120 ppt) and temperatures (−8 and +15°C) to investigate changes in fluorescence as a result of the experimental conditions; no such changes were observed. Rate values were scaled to bacterial concentration in the harvested culture pre-filtration to enable cell-specific comparisons across laboratory assays and between lab and field assays. Three-way ANOVA and Student's *t*-tests were used to determine significant differences in rate where applicable using a p-value cutoff of 0.05, with statistical analyses performed using the 'native' package in R Studio (v 1.4.1106).

2.5. Bacterial counts

Manual enumeration of bacteria was performed using epifluorescence microscopy as described in previous studies (Marx et al. 2009, Ewert & Deming 2014). Briefly, bacteria fixed with 2% final concentration formaldehyde were filtered over a black polycarbonate membrane filter (Sigma). Filters were stained with 4′,6′-diamidino-2-phenylindole (DAPI) in ASW at 20 µg ml^{-1} and then viewed on a Zeiss Universal epifluorescent microscope, counting at least 200 cells per field and 20 fields per sample.

3. RESULTS

3.1. EEA of *Cp*34H and *P*7E grown at optimal conditions

After growth at conditions for maximum cell yield (−1°C, 35 ppt; Huston 2003), free EEA for *Cp*34H measured at −1°C across a salinity profile (17.5, 35, 55, 75, 90, 120 ppt) showed a dependence on salinity,

with the highest rate at 90 ppt and lowest rate at 17.5 ppt; i.e. 1.5×10^{-7} and 0.65×10^{-7} nM MCA-L h^{-1} $cell^{-1}$, respectively (Fig. 1). Activity was still detectable at 120 ppt, the highest salinity tested. The same constant-temperature experiment performed on *P*7E resulted in a different pattern. Not only was enzyme activity roughly an order of magnitude lower overall, with a maximum rate of activity of 0.2×10^{-7} nM MCA-L h^{-1} $cell^{-1}$, but activity showed no apparent pattern in relation to salinity. EEA with *P*7E was negligible at 120 ppt, the highest salinity tested. Integrated across all salinities, *Cp*34H showed significantly higher average activity at −1°C than *P*7E ($p = 0.0001$), while *P*7E showed significantly higher activities at 8°C ($p = 0.04$) and 15°C ($p = 0.03$) according to a Student's *t*-test.

When grown at optimal conditions (−1°C and 35 ppt) but assayed at different temperatures (−4, 8, and 15°C), free EEA from *P*7E demonstrated a stronger positive temperature dependence and higher overall activity than *Cp*34H. While the overall EEA of both strains increased at higher temperatures, the maximum EEA reached by *Cp*34H did not differ significantly ($p > 0.05$) from that measured at −1°C, staying within the range of 1.5 to 2.0×10^{-7} nM MCA-L h^{-1} $cell^{-1}$. The maximum EEA of *P*7E, however, was significantly increased ($p = 0.03$) by temperature, jumping from 0.2×10^{-7} nM MCA-L h^{-1} $cell^{-1}$ at −1°C to 3.3×10^{-7} nM MCA-L h^{-1} $cell^{-1}$ at 15°C (Fig. 2). Both strains showed maximum EEA at salinities above their optimal growth salinity: *Cp*34H at about 90 ppt, and *P*7E at about 55 ppt.

3.2. EEA of *Cp*34H grown at non-optimal conditions

When produced at non-optimal growth conditions, the EEA of *Cp*34H showed dependence on growth temperature. When cells were grown at optimal salinity (35 ppt) but different temperatures (−8, −4, or 8°C) and the resulting enzymes were assayed across a temperature and salinity range (−8, −4, −1, 8, 15°C; 17.5, 35, 55, 75, 90, 120 ppt), maximum EEA rates were observed when the enzymes were assayed at the warmest temperature of 15°C but produced at either temperature growth extreme of −8 or +8°C (5.5×10^{-7} and 4.6×10^{-7} nM MCA-L h^{-1} $cell^{-1}$, respectively) (Fig. 3).

When *Cp*34H was instead grown at optimal growth temperature (−1°C) but non-optimal salinity (55 or 70 ppt), maximal EEA was observed by cells grown at the higher salinity of 70 ppt (Fig. 4). This maximum rate of EEA (1.3×10^{-6} nM MCA-L h^{-1} $cell^{-1}$ at both 15°C and 55 ppt) was roughly an order of magnitude higher than rates of EEA produced by cells grown at a lower salinity of 55 ppt (2.3×10^{-7} nM MCA-L h^{-1} $cell^{-1}$, assayed at 15°C and 70 ppt) or of 35 ppt (as stated above, 1.5×10^{-7} nM MCA-L h^{-1} $cell^{-1}$, assayed at 15°C and 90 ppt).

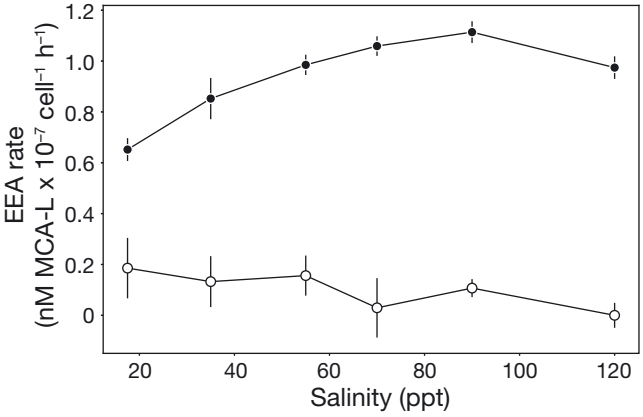

Fig. 1. Rate of extracellular enzyme activity (EEA) at −1°C as a function of salinity. Data represent the average rate of EEA for both *Colwellia psychrerythraea* strain 34H (*Cp*34H; ●) and *Psychrobacter* strain 7E (*P*7E; ○) at each salinity. Both bacterial strains were grown at −1°C and 35 ppt. Error bars indicate ± SD of replicate samples (n = 3). MCA-L: L-leucine-7-amido-4-methylcoumarin hydrochloride

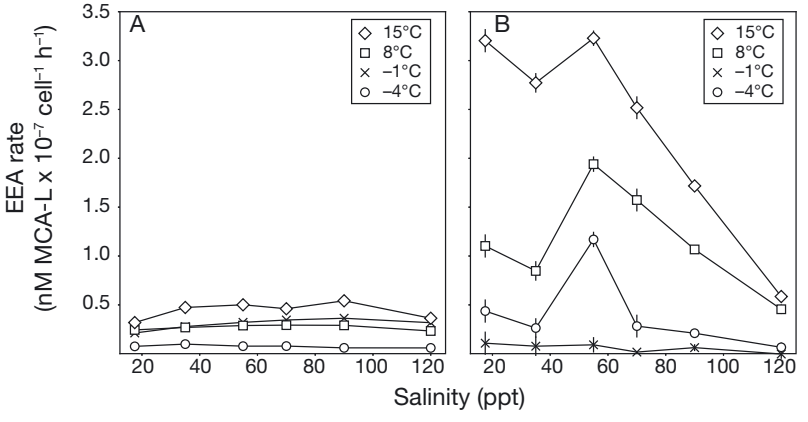

Fig. 2. Rate of extracellular enzyme activity (EEA) as a function of temperature and salinity given the same growth conditions. Data represent the EEA rates for both (A) *Cp*34H and (B) *P*7E across a temperature spectrum from −4 to +15°C and salinity spectrum from 17.5 to 120 ppt when the EEs were produced by organisms grown at −1°C and 35 ppt. Symbols represent the average rate (n = 3) for each salinity assayed at temperatures of 15°C, 8°C, −1°C, or −4°C. Error bars indicate ± SD of the triplicate samples; where not visible, error bars are smaller than the symbols

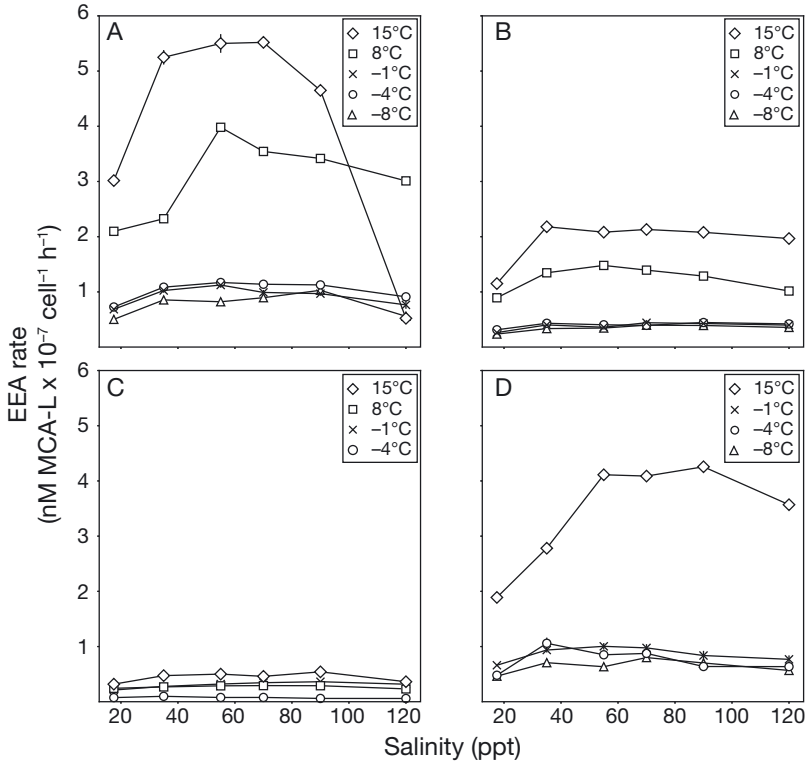

Fig. 3. Rate of extracellular enzyme activity (EEA) as a function of temperature and salinity given different growth temperatures. Data represent the EEA rates for *Cp*34H across a temperature spectrum between −8 and +15°C and salinity spectrum from 17.5 to 120 ppt after growth (EE production) at 35 ppt and 4 different temperatures: (A) −8°C, (B) −4°C, (C) −1°C (included from Fig. 2 for visual comparison), and (D) 8°C. EEA assay temperatures were 15°C, 8°C (assay data at 8°C unavailable for cells grown at 8°C in panel D), −1°C, −4°C, and −8°C (data for −8°C unavailable for cells grown at −1°C in panel C). Error bars indicate ± SD of replicate samples (n = 3); where not visible, error bars are smaller than the symbols

A 3-way ANOVA performed using R Studio to compare effects of treatment temperature and treatment salinity on EEA of *Cp*34H grown under different temperature conditions revealed statistically significant effects of treatment temperature ($p < 0.0001$) but no interactions. A 3-way ANOVA of effects of treatment conditions on EEA of *Cp*34H grown under different salinity conditions demonstrated significant effects of treatment temperature ($p = 4.78 \times 10^{-11}$) and growth salinity ($p < 0.0001$), as well as significant interactions between treatment temperature and growth salinity.

3.3. EEA in environmental samples

Field measurements of EEA showed activity across all samples and conditions (Table 1): under-ice water rates of 0.03–0.1 nM MCA-L h^{-1}, SML rates of 0.3–0.6 nM MCA-L h^{-1}, and sea-ice rates of 0.7–3.7 nM MCA-L h^{-1}. As sea-ice and frozen SML EEA values were calculated to bulk volume rather than brine volume, EEA may be higher *in situ* than the values provided here. Considering cell-specific values, sea-ice core samples demonstrated the highest EEA rates (maximum of 6.4×10^{-6} nM MCA-L h^{-1} $cell^{-1}$), measured at near *in situ* conditions (−1°C, with a measured salinity of 19.5 ppt). The sea-ice EEA maximum was >30 times higher than EEA in seawater at −1.8°C and 32 ppt (0.2×10^{-6} nM MCA-L h^{-1} $cell^{-1}$) and about 6 times higher than EEA in SML at −1°C and 8 ppt (1.0×10^{-6} nM MCA-L h^{-1} $cell^{-1}$). Refrozen sea-ice cores, measured over a 10 d period at −10°C and with a calculated brine salinity of 142 ppt, retained higher activity compared to re-frozen SML. However, the re-frozen core activity was still lower than EEA in unfrozen (melted) ice cores, at 1.7×10^{-6} nM MCA-L h^{-1} $cell^{-1}$. Warming increased the EEA rate in the underlying seawater to 0.5×10^{-6} nM MCA-L h^{-1} $cell^{-1}$, while refreezing and then re-thawing SML decreased the EEA rate to 0.8×10^{-6} nM MCA-L h^{-1} $cell^{-1}$, which was near that of warmed seawater. Finally, endpoint measurements of frozen SML (−10°C, calculated brine salinity of 100 ppt) proved challenging; replicate tubes were lost shipboard and only 1 measurement was made, which demonstrated a substantially decreased rate (0.1×10^{-6} nM MCA-L h^{-1} $cell^{-1}$). A 3-way ANOVA, performed in R Studio, comparing sample type, sample temperature, and sample salinity for each measurement, showed significant effects by sample type ($p = 0.0072$), but no significant interactions. A full list of field measurement rates and their errors is given in Table 1.

4. DISCUSSION

This study presents a new understanding of the limits of bacterial EEA under the combined sea-ice conditions of low temperature and high salinity. Primarily, these results have demonstrated the ability of

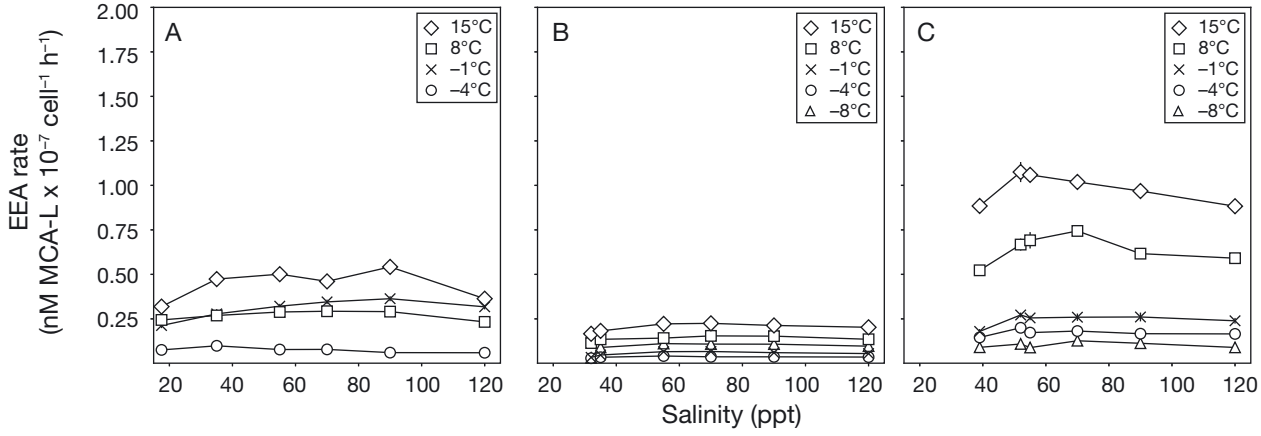

Fig. 4. Rate of extracellular enzyme activity (EEA) as a function of temperature and salinity given different growth salinities. Data represent the EEA rates for *Cp*34H across a temperature spectrum between −8 and +15°C and salinity spectrum between 17.5 and 120 ppt after growth at −1°C and (A) 35 ppt, (B) 55 ppt, or (C) 70 ppt. Symbols represent the average rate for each salinity assayed at temperatures of 15°C, 8°C, −1°C, −4°C, and −8°C (data for −8°C unavailable for cells grown at 35 ppt in panel A). Error bars indicate ± SD of replicate samples (n = 3); where not visible, error bars are smaller than the symbols. Data for EEA when *Cp*34H was grown at −1°C and 35 ppt (A, from Fig. 2) are included for visual comparison. The difference in minimum test salinity for the 55 and 70 ppt experiments (in B and C) is a result of maintaining constant organic content (for growth) through dilution

Table 1. Extracellular enzyme activity (EEA) data from field samples. The averages (±SD) of measured EEA rates (n = 3, unless noted) in samples of bottom sea ice, under-ice water, and the sea-surface microlayer are displayed alongside experimental temperatures and salinities. A 3-way ANOVA revealed significant difference by sample type (p = 0.00717). MCA-L: L-leucine-7-amido-4-methylcoumarin hydrochloride

Sample type (treatment)	Temperature (°C)	Salinity (ppt)	EEA rate (×10^{-6} nM MCA-L h^{-1} cell^{-1})
Under-ice water	−1.8	32	0.150 ± 0.18
Under-ice water (warmed)	6	32	0.532 ± 0.09
Sea-surface microlayer	−1	8	1.01 ± 0.02
Sea-surface microlayer (frozen)	−1.8	8	0.832 ± 0.076
Sea-surface microlayer (frozen, endpoints)	−10	100	0.15[b]
Bottom sea ice (thawed)	−1	19.5	6.41 ± 0.15
Bottom sea ice (thawed and refrozen)	−10	142[a]	1.66 ± 0.04

[a]Salinities of these 2 frozen samples were calculated by applying the equations of Cox & Weeks (1983) to bulk salinity measurements of unfrozen (melted) samples
[b]n = 1

psychrophilic bacteria to produce EEs when grown at low temperature (−8°C) or high salinity (70 ppt) and maintain activity under such conditions both in the laboratory and within sea-ice, seawater, and SML field samples under manipulated conditions. EE production and activity under these conditions have implications for ecological processes and applications to biotechnology. Primarily, these data demonstrate that psychrophilic bacteria are able to mediate the hydrolysis of extracellular organic compounds under extremes of temperature and salinity by means of their EEs, presumably in turn supporting their requirements for substrate in order to maintain metabolic activity or grow in extreme environments. Free EEs in the sea-ice environment may also serve as a 'digestor' of large dissolved organic compounds within sea-ice brines and work to prime organic material for uptake by seawater communities at the onset of spring ice melt. Likewise, free EEs that can operate under extreme conditions could be made useful in applied research, including industrial processes that require cold or high-salt conditions (Karan et al. 2020) and potential applications requiring use of an organic solvent that causes enzymes to experience low water activity, as they would under cold hypersaline conditions.

Extracellular hydrolytic activity of psychrophilic and psychrotolerant organisms has been character-

ized previously in isolates from a variety of cold environments including Arctic seawater and Arctic marine sediments (Srinivas et al. 2009, Prasad et al. 2014). While most of these studies involved warm conditions relative to the original environment, often above 0°C and more commonly above 4°C, their rates of peptidase activity are comparable to those of our laboratory samples evaluated at subzero temperatures. Conversely, *in situ* characterizations of enzyme activity are relatively uncommon in natural sea-ice communities (Helmke & Weyland 1995, Huston et al. 2000, Deming 2007, Yu et al. 2009) or with sea-ice isolates (Q. Wang et al. 2005, X. Wang et al. 2018). Our results are comparable to previous observations of EEA rates by Helmke & Weyland (1995), which ranged between 0.1 and 51.1 nM MCA-L h^{-1} at −1°C, with cell concentrations of similar magnitude to ours (mostly 10^4 cells ml^{-1}) though highly variable. Our values are somewhat lower than those of Huston et al. (2000), which ranged between about 100 and 350 nM MCA-L h^{-1} at −1 to 6°C, although they reported similar differences in EEA rate between ice-core and under-ice water samples.

Notably, the values we report are also similar to EEA measurements from open water marine environments, which largely range between 5 and 80 nM MCA-L h^{-1} (for a review, see Arnosti 2011), including within high-latitude Arctic waters (e.g. Huston & Deming 2002, Kellogg & Deming 2014, Balmonte et al. 2018). However, the fluorescence-based activity assay used here (and in many other environmental studies) cannot distinguish the cause of differences in EEA, which may result from enhanced activity of individual enzymes (i.e. the total EE concentration does not change as a result of growth conditions, but rather each enzyme is more active) or from greater overall concentration of enzymes (i.e. the growth conditions stimulate production of more EEs, which may mask individual enzyme efficiency). This distinction is especially important when comparing EEA from EEs produced under different bacterial growth conditions, which often stimulates changes in concentration of EEs produced (see Section 4.1). Any observed differences in enzyme activity rate could be the result of higher or lower individual enzyme activity or higher or lower concentration of enzymes.

4.1. Patterns of activity suggesting psychrophilic nature

For both *Cp*34H and *P*7E, the pattern of maximal rates of EEA at supra-optimal growth temperatures is consistent with the well-established understanding of thermal dependence of enzyme activity. For *Cp*34H, previous characterizations of its leucine aminopeptidase when grown at 8°C demonstrated maximum activity at 19°C and reduced activity at lower temperatures (about 70% of maximum at 15°C and about 15% of maximum at −1°C; Huston et al. 2004), although characterization of an aminopeptidase from another sea-ice *Colwellia* showed a much higher temperature optimum (35°C, Wang et al. 2005). A similar magnitude reduction in rate between 15 and −1°C to that observed by Huston et al. (2004) was found in our study; both are consistent with reduction in enzyme activity due to temperature effects on chemical reactions when an Arrhenius equation is applied. Because the enzyme concentration was not quantified in this experiment, accurate kinetic parameters cannot be deduced, nor can we comment on the possibility of a temperature-driven reprogramming of gene expression or protein modification. However a thermal effect coefficient can be inferred from enzymes assayed under different temperatures if produced under the same growth conditions. The observed change in enzyme activity as a function of experimental temperature is consistent with Q10 thermal effects, assuming a Q10 coefficient between 2 and 3 and constant enzyme production under the same growth conditions.

The pattern of EEA by *Cp*34H as a function of growth condition demonstrates that *Cp*34H can produce functional EEs even when grown at the most extreme temperature tested, −8°C (and 35 ppt). Previous observations recorded increased EEA rates for *Cp*34H when grown at cold but less extreme temperature (−1°C) compared to a warmer temperature (8°C), likely due to increased EE production at the lower temperature (Huston et al. 2000).

Measurements of salinity effects on EEA have been performed on EEs produced by bacteria isolated from cold environments (e.g. amylase, Qin et al. 2014; esterase, Tchigvintsev et al. 2015), including from sea ice (amylase, Wang et al. 2018), but fewer have been performed on cold-active leucine aminopeptidases or proteases (Lei et al. 2017, Salwan et al. 2020). Patterns of enzyme sensitivity to salt from these studies, as well as studies of non-cold-active enzymes, are consistent with our observations of maximal EEA rates at supra-optimal salinities for both *Cp*34H and *P*7E (i.e. at 55–90 ppt). Although enzymes can display a variety of salinity effects, moderately high salinity (e.g. between 0.5 and 2.0 M NaCl, as observed by Qin et al. 2014 and Wang et al. 2018) can enhance enzyme activity in some prokary-

otes by stabilizing protein structures and interactions (Ortega et al. 2011, Sinha & Khare 2014). The overall higher level of activity of enzymes produced by *Cp*34H when grown at the supra-optimal salinities of 55 and 70 ppt suggests that the enzymes may indeed be charge-stabilized by the higher salinity, though we cannot exclude the possibility of excess EE production at these salinities. However, salinities well beyond the tolerance of an organism can disrupt protein structure, reducing EEA rates. Such effects have been seen in studies of enzymes from other marine bacteria (Qin et al. 2014, Wu et al. 2015, De Santi et al. 2016, Wang et al. 2018). A disruptive effect likely explains the lower rates of EEA observed at 120 ppt for *Cp*34H, which is well beyond the maximum growth salinity of *Cp*34H (although the upper salinity bound for activity or survival of *Cp*34H is not known). Other organisms show monotonically decreasing enzyme activity with increased salt concentrations (Takenaka et al. 2018), such as observed with *P*7E, but our results with *P*7E are notable given the wide salinity range for its growth. Given that our experiments assayed free EEs and not attached EEs, an open question is whether *P*7E employs a different production strategy than *Cp*34H, favoring attached over free EEs. Indeed, observations in marine systems have shown that the fraction of attached enzyme activity can vary greatly within an environment (Baltar 2018).

Although the fluorescence assay used in this study may not distinguish between enhanced activity rates of individual enzymes and a higher concentration of enzymes, the enhanced EEA of *Cp*34H when grown at the most extreme temperature tested (−8°C, well below maximal growth rate and yield temperatures) is likely the result of the latter; many cold-adapted bacteria reach maximal EE production at temperatures below their optimal growth temperatures (Buchon et al. 2000). Such behavior may represent a metabolic trade-off between growth and acquisition of utilizable organic matter (Ramin & Allison 2019). In cold marine environments, canonical thought suggests that bacteria require higher substrate concentration to enable activity and growth (Pomeroy & Wiebe 2001). Previous studies have demonstrated that free EEs can liberate enough organic substrate to sustain bacterial growth (Vetter et al. 1998, Vetter & Deming 1999). Enhanced enzyme production may thus help to balance this resource need within sea-ice brines, where characteristic extremes of temperature and salinity already act to reduce growth rates.

Likewise, the fluorescence assay used in this study cannot determine variable structures or isomers of enzymes that interact with the substrate, which may obscure changes in measured EEA as a result of differences in enzymes or post-modification of enzymes as a function of temperature or salinity. Indeed, previous work by Steen et al. (2015) has shown that extracellular peptidases show affinity for a multitude of amino acids.

4.2. EEA at the seawater/sea-ice interface

Observations from field samples and freeze–thaw cycle experiments showed that rates of EEA increased from seawater to SML to sea ice (with significant differences by sample type), reflecting the different physical and biological characteristics of each niche. Underlying seawater, with the lowest rates of activity, is the most dilute of these environments, whereas both the SML and sea ice can be expected to contain higher concentrations of organic matter. During growth in dilute (but non-starving) nutrient conditions, bacteria are less likely to produce EEs (Cezairliyan & Ausubel 2017). For the SML, previous investigations at temperate latitudes have demonstrated EEA within this environment, but such measurements are sparse (Kuznetsova & Lee 2001, Mustaffa et al. 2017, Perliński et al. 2017). Our study is unique for demonstrating EEA in an Arctic SML and nearby sea ice and tracking EEA through a freeze–thaw cycle that mimics natural conditions.

EEs likely retain activity through the freezing and concentration process, even if some of the bacteria producing these enzymes may not. Free enzymes are known to persist in cold water (Steen & Arnosti 2011) and be stabilized by bacterial extracellular polysaccharide substance (EPS) (Huston et al. 2004), which can be selectively entrained into sea ice during formation (Ewert & Deming 2011). EPS, produced copiously in sea ice by algae (Krembs et al. 2002, Meiners et al. 2003), are also produced on a smaller scale by bacteria, as shown in simulated sea-ice brines when temperature was decreased to −8°C (Marx et al. 2009). The potential for EPS to extend the life-time of free EEs present in sea ice adds to the concept that such enzymes, persisting unlinked to their producers, represent activity of the 'living dead' (Baltar 2018). An extended lifetime, in addition to ongoing EE production, may lead to accumulation of free EEs, including 'unlinked' enzymes long removed from their producers.

Within brines, enzymes experience increased viscosity as temperature drops and salinity increases (Cox & Weeks 1975), limiting diffusion which affects

strategies of EE production. By their nature, EEs are a community good, meaning degraded substrate will diffuse to benefit the closest cell regardless of which cell produced the enzyme (Vetter et al. 1998). In models of well-mixed, low-viscosity environments, this effect leads to extinction of EE-producing cells and the proliferation of 'cheater' cells, those that do not produce EEs but reap their benefits; conversely, under a high-viscosity regime with spatial structuring, a mixed community of producer and cheater cells proliferates (Wakano et al. 2009, Allison et al. 2014). Given the increased viscosity of sea-ice brines, a mixed community of cheaters and enzyme producers therefore seems likely in this environment. This interpretation implies active production of EEs within sea ice, a conclusion supported by our observation of EEA in cultures of *Cp*34H grown at –8°C. However, the diffusion regime suggests that free EEs offer a low return for producers and cheaters alike because of higher viscosity conditions, especially for motile cells (Traving et al. 2015). Given that motility has been observed in sea-ice brines under near *in situ* conditions (Lindensmith et al. 2016) and in *Cp*34H under analogue brine conditions (Junge et al. 2003, Wallace et al. 2015, Showalter & Deming 2018), attached EEs may be a more beneficial strategy in sea ice, a testable hypothesis for future work.

4.3. Environmental implications

While EEA is often considered the rate-limiting step for organic carbon cycling in the marine environment, our results indicate that within the brines of frozen seawater, EEA may not be the primary rate-limiting factor. Slower bacterial growth and metabolic activity rates preclude a need for rapid hydrolysis of large-sized organic material, especially if free enzymes are abundant due to freeze-in and slow decay rates. Rather, enzyme activity in sea-ice brines may be most ecologically relevant when considering the composition of the organic material present and phase transitions during ice growth and melt.

Much organic matter within sea ice is carbohydrate, largely as a result of EPS production for cryoprotection and osmoprotection, although sea-ice brine pockets can also be enriched in proteinaceous material relative to source seawater (Thomas et al. 2001, Underwood et al. 2010, Stedmon et al. 2011, Müller et al. 2013). While the aminopeptidase assay of this study would not measure enzymatic degradation of carbohydrate-rich materials, the bacterial production of active proteolytic enzymes under extreme conditions suggests that sea-ice communities likely have the ability to produce carbohydrate-degrading and other enzymes as well; indeed, chitobiase activity has been documented along with aminopeptidase activity in sea ice (Huston et al. 2000).

Active hydrolysis within sea ice brines could increase the relative proportion of low molecular weight organics to the benefit not only of sea-ice communities but also SML and seawater communities during ice melt. In effect, high rates of EEA within sea-ice brines could define sea ice as a 'digestor,' with particular benefits for members of a community less well adapted to extreme temperature or salinity. This role may be especially important where metabolic streamlining has led to a narrower range of functional enzymes (Steen & Arnosti 2014); long periods of enzymatic digestion in sea-ice brines could circumvent this apparent limitation and decrease community response time to organic input during melt season. Indeed, organic material from sea ice less than 100 kDa in size has been shown to promote rapid growth in seawater bacteria after ice melt (Underwood et al. 2019).

In contrast, the SML, which also contains higher concentrations of organic matter than underlying seawater, is enriched in amino acids rather than carbohydrates (Engel et al. 2018). While peptidase activities, as well as carbohydrase and lipase activities, have been confirmed in the SML in past studies, these enzyme activities were measured at relatively warm temperatures (>4°C; Kuznetsova & Lee 2001) or unspecified temperatures (Mustaffa et al. 2017, Perliński et al. 2017). Our work demonstrates that EEA in the SML proceeds both at subzero temperatures and immediately following thawing of frozen SML samples, suggesting that the involved EEs can survive the thaw–freeze transition and continue to be active within sea ice. EEA within the SML at low temperatures may also have atmospheric implications: organic material from the ocean surface can serve as ice-nucleating material when of the proper size (Chance et al. 2018). The ice-nucleating potential of components of the SML is especially relevant in the Arctic, where future cloud cover is a large unknown.

Further characterizations of enzyme activity under extreme conditions are warranted. As highlighted by Arnosti (2011), the standard method of EEA measurement (Hoppe 1983) presents an incomplete picture of environmental processes with respect to substrate specificity and degradation of complex compounds such as EPS. Quantifying enzyme production under extreme conditions may also enhance our understanding of which organisms actively produce en-

zymes under specific environmental conditions and which organisms reap the benefit. The results presented here serve to expand our understanding of bacterially mediated carbon cycling under the combined extremes of low temperature and hypersalinity that characterize sea ice. In demonstrating EEA to −8°C and 120 ppt salts in the laboratory, and activity to −10°C and up to 142 ppt salts in field samples, we present a potential mechanism for DOM degradation within winter sea ice, potentially serving as a primer for spring communities at the onset of ice melt by increasing the fraction of low molecular weight organic matter readily available for uptake.

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